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14. **ABSTRACT**
Resveratrol, a grape-derived polyphenol, is a chemopreventive agent shown to suppress androgen-dependent and -refractory prostate cancer (CaP) cell growth, and inhibit prostate specific gene expression. To further elucidate its anti-CaP properties, we advance the hypothesis that resveratrol interacts with specific cellular target proteins, denoted RTPs. This project aims to identify and purify RTPs. Our first approach was to test the ability of [3H]resveratrol to form stable complexes with RTPs based on retention on nitrocellulose filters or chromatography on gel filtration columns. Feasibility of this approach was tested using extracts prepared from LNCaP and PC-3 cells. This approach was unsuccessful possibly due to technical limitations, such as: scarcity of RTPs, low specific activity of labeled resveratrol and difficulties in forming stable [resveratrol.RTP] complexes. An alternative approach involved chemically immobilizing resveratrol on epoxy-activated agarose to generate a biospecific affinity matrix for isolating and purifying RTPs from cell extracts. We have named this affinity chromatography approach ligand-select proteomics (LSP) as it affords a panoramic display of proteins having different binding affinities to resveratrol. By combining LSP with MALDI-TOF mass spectrometry, we have identified dihydronicotinamide riboside:quinone reductase (NQO2) as a distinct RTP from LNCaP and PC3cells.

15. SUBJECT TERMS
Resveratrol targeting proteins, affinity column chromatography

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INTRODUCTION

This progress report represents an updated summary of our research activities along the two tasks we have proposed and outlined in the Statement of Work (SOW). We also have provided explanations to the issues raised by the reviewer in a previous report we submitted in November, 2005.

The primary focus of Task 1 in SOW was to test the hypothesis that proteins capable of interacting and binding resveratrol, denoted RTPs, exist in both androgen-dependent and –independent prostate cancer cells. Our experimental strategy as proposed in Task 1 involved determining ability of [^{3}H]resveratrol to form stable complexes with RTPs. We suggested that formation of such complexes might be monitored by (i) retention on nitrocellulose filters, and (ii) by chromatography on gel filtration columns. We performed pilot experiments to test the feasibility of these approaches using extracts prepared from LNCaP and PC-3 cells. Exceedingly low but reproducibly observed uptake of radioactive resveratrol into LNCaP cells was found. Somewhat increased uptake was also observed in PC-3 cells. In neither cell types, however, was uptake sufficient for demonstration of nitrocellulose retainable complexes. The lack of success of our proposed approaches may be attributable to: scarcity of RTPs; instability of [resveratrol.RTP] complexes; low binding affinity between resveratrol and RTPs; low specific activity of [^{3}H] resveratrol.

As an alternative approach, we proceeded to perform experiments outlined in Task 2 of SOW. The major emphasis of this task involved attempts to covalently couple resveratrol to an immobile matrix. This was accomplished by immobilizing resveratrol on epoxy-activated agarose, resulting in a ligand (resveratrol)-linked affinity matrix with capability to interact with and specifically retain RTPs from prostate cancer cell extracts. A purification scheme, involving sequential elution with high salt (0.35 M followed by 1.0 M NaCl), 1 mM ATP, and finally 1-2 mM resveratrol, for RTPs having different binding affinities for resveratrol was developed empirically. We have named this approach ligand-select proteomics (LSP). By combining LSP with MALDI-TOF mass spectrometry, we have identified dihydronicotinamide riboside:quinone reductase as a distinct RTP from LNCaP and PC-3 cells.

BODY

Task 1. To demonstrate proteins capable of binding resveratrol in prostate cancer cells.

Task1a. Develop a nitrocellulose filter-binding assay for resveratrol-binding protein(s) using androgen-dependent LNCaP cells.

Rationale for approach: The basis for this approach was identical to what was used previously in studies on induction of enzymes by interferon — specifically, 2',5'-oligoadenylate dependent ribonuclease L (RNase L) and the double-stranded RNA-activated 2',5'-oligoadenylate synthetase (2-5AOS). The PI had utilized specificity and affinity between novel bioactive ligands and cellular proteins as the basis for the identification, isolation and characterization of such targeting proteins. Both RNase L and 2-5AOS were characterized with assays involving binding of radioactive ligands to crude cell extracts; formation of ligand:enzyme complex was determined by retention on nitrocellulose membranes (1-7). However, binding was not always successful especially in cases when low specific activity radiolabeled ligand was available, as well as in extracts containing low abundance of putative binding target protein(s).

Our attempts to demonstrate the ability of [^{3}H]resveratrol to form stable complexes with RTPs whose presence may be demonstrated and quantitated by retention on nitrocellulose filters were unsuccessful using LNCaP cell extracts. Uptake studies to determine the amount of [^{3}H] resveratrol taken into cells provided an explanation for the difficulties we encountered. As illustrated in Table 1 below, very little [^{3}H] resveratrol was taken up by LNCaP cells. Typically only 300-750 cpm resveratrol was found in 0.1 ml cell extracts containing 200-300 μg total protein, suggesting that performance of this task was unlikely to be feasible and in all probability technically untenable. Nevertheless, significantly above background radioactivity in association with lysed LNCaP cell extracts was repeatedly observed, thus providing support for our hypothesis that resveratrol targeting proteins are present in CaP cells.

Table 1. Amount of [^{3}H] resveratrol bound to LNCaP cell extracts

LNCaP	Labeling condition	Amount label	Cytosol (cpm/100 μl)	pellet
Control-2 day	1 h (pulse)	60 μl (1.8 μCi)	350	150
Control-3 day	3 h (pulse)	60 μl	750	0
Control-4 day	3 h (pulse)	45 μl	300	0
Control-4 day	3 days (continuous)	60 μl	450	0

[^{3}H] resveratrol: 30μCi/ml H_2O; 7.5 μCi/250 μl (specific activity, 15 C*i*/mmol)

Conclusions: The above results suggest that development of a binding assay as an approach to monitor and ascertain the presence of resveratrol binding protein is unlikely to yield meaningful results.

Task 1b. Confirm binding of resveratrol to target proteins using gel-filtration column chromatography

This task was not performed and abandoned due to inadequate uptake of [^{3}H] resveratrol, as detailed above, into cell extracts. The next phase of our focused research

stemmed from the rationale that experimental demonstration of targeting proteins for resveratrol may be significantly improved by first developing a ligand (resveratrol)-linked affinity column as we originally proposed in Task 2a. Our hypothesis is that putative targeting proteins of resveratrol can "dock" with resveratrol at distinct "motifs". Notably, interaction between resveratrol and its cellular mediators, which we denote resveratrol targeting proteins (RTPs) may occur via its aromatic stilbene core and hydrophilic side groups. Although such aromatic centers are ubiquitous and only weakly interactive, they are important "sensor/detector" elements in biological systems. Development of such an affinity matrix also will afford a one-step enrichment of low abundance proteins of interest. Details of our progress in this objective are described in detail as part of Task 2 below.

Task 1c. Perform binding assays using extracts prepared from androgen-independent PC-3 cells

In addition to LNCaP cells, we also performed uptake studies of radioactive resveratrol using PC-3 cells. Compared to LNCaP cells, somewhat greater amount of labeled resveratrol was taken up and became bound to proteins in the cytosol, as shown by results in Table 2.

Table 2. Amount of [^{3}H] resveratrol bound to PC-3 cell extracts

PC-3	Condition for labeling	Amount label	Total (cpm/100 µl)	Net uptake (cpm/100 µl)	Total (cpm/300 µl)	Net uptake (cpm/100 µl)
Control-2 day	label for ~ 0 min	60 µl	1297, 1329		4024, 4090	
Control-2 day	label for 15 min	60 µl	1401, 1315	45	4291, 4252	72
Control-2 day	label for 30 min	60 µl	1894, 1906	598	5897, 5705	582

Response to technical issues raised by reviewer on p2- last sentence, paragraph 1, of an earlier report submitted in November, 2005

Reviewer's comment: "Even more troubling is the observation that the controls in which no [^{3}]H-resveratrol was applied also showed [^{3}]H incorporation."

Response: We did add label [^{3}]H-resveratrol into cultured cells which were immediately harvested and processed as data for time = 0 min to be used as the baseline value for [^{3}]H-resveratrol uptake (Table 2). These experiments showed that there was considerable background. Net uptake of resveratrol into PC-3 cells was calculated using the equation:

For 15 min uptake = $cpm_{Time=15\ min} - cpm_{Time=0\ min}$

For 30 min uptake = $cpm_{Time=30\ min} - cpm_{Time=0\ min}$

The results in Table 2 show that uptake of resveratrol in PC-3 cells was initially slow since the amount of [^{3}]H-resveratrol bound to cell extracts at 15 min was barely above the background. By contrast, [^{3}]H-resveratrol became more substantially bound at the 30 min time point.

Since our previous studies on the interferon-responsive RNase L and 2-5OAS showed that expression of binding protein could be upregulated by exposure to interferon (4-7), we considered the possibility that expression of resveratrol targeting proteins

RTPs, if present, may also increase by treatment with resveratrol, which could manifest as an increase in [^{3}]H-resveratrol uptake. Accordingly, PC-3 cells were treated with varying doses of unlabeled resveratrol for 2 days, after which cells were washed to remove unlabeled resveratrol. Resveratrol-treated cells processed in this manner were then labeled with [^{3}]H-resveratrol and uptake of [^{3}]H-resveratrol into resveratrol-treated PC-3 cells was compared to untreated cells using the same approach shown in Table 2 and presented in Table 3..

For 15 min uptake = $cpm_{\text{Time=15 min for resveratrol treated cells}} - cpm_{\text{Time = 0 min for resveratrol-treated cells}}$

For 30 min uptake = $cpm_{\text{Time= 30 min for resveratrol treated cells}} - cpm_{\text{Time= 0 min for resveratrol treated cells}}$

Table 3. Uptake of [^{3}H] resveratrol using resveratrol-treated PC-3 cell extracts

PC-3	Labeling Condition	Amount label	Total (cpm/100 µl)	Net uptake (cpm/100 µl)	Total (cpm/300 µl)	Net uptake (cpm/100 µl)
2µM resveratrol-2 day	label for ~ 0 min	60 µl	1761, 1765		5461, 5191	
2µM resveratrol-2 day	label for 15 min	60 µl	1858, 1759	45	5353, 5431	23
2µM resveratrol -2 day	label for 30 min	60 µl	3426, 3404	1652	9973, 10170	1582

The results in Table 3 once again showed a lag of about 15 minutes regarding uptake of [^{3}]H-resveratrol, similar to what was observed for untreated PC-3 cells (see Table 2). However, additional interesting observations surfaced from these experiments. Namely, when PC-3 cells were first treated for 2 days with 2 µM resveratrol, the uptake of [^{3}]H-resveratrol was stimulated by 276% (1652 cpm for resveratrol-treated cells/598 cpm for control cells, both labeled for 30 min), suggesting that treatment of PC-3 cells by resveratrol can increase the expression of proteins having affinity for resveratrol.

Response to technical issues on p2 regarding the first concern from reviewer.

Reviewer's comment: "For example, in Figure 1, less resveratrol was incorporated into cells when higher concentrations of resveratrol were applied to the culture, i.e., 5 micromolar (µM) versus 10 µM. Heedless of these results, the PI uses even more resveratrol (25 µM) for further experiments."

Response: Reviewer made a correct and astute observation. Indeed, compared to untreated controls, less [^{3}]H-resveratrol was incorporated into PC-3 cells first treated for 2 days with still higher concentrations of resveratrol (10 µM) prior to the labeling studies (see Fig. 1C of report submitted in November 2005). Two possible explanations may be offered. First, targeting proteins RTPs may already become saturated with unlabeled resveratrol during the treatment phase and this effectively interfered with the subsequent uptake of [^{3}]H-resveratrol. Another plausible but somewhat less likely possibility is that

treatment with a dose of 10 μM effectively suppressed the expression of resveratrol target proteins RTPs, whereas treatment by 2 μM induced an increase in RTP expression.

Our preliminary studies using normal prostate epithelial cells (PrEC, see Fig. 1B of 2005 report) showed modest uptake of [^{3}]H-resveratrol, with a slight stimulation of uptake possibly observed during the first 5 minutes when PrEC was pretreated for 2 days with 5 or 10 μM resveratrol.

In normal prostate stromal cells (PrSC, see Fig. 1A of 2005 report), linear uptake of [^{3}]H-resveratrol for up to 20 minutes was observed. Pretreatment by resveratrol also stimulated the uptake of [^{3}]H-resveratrol in PrSC.

Conclusions: Uptake of [^{3}]H-resveratrol is dependent on whether normal or tumor prostate cells are used. Comparison of its uptake in LNCaP and PC-3 cells shows a more robust incorporation of [^{3}]H-resveratrol in PC-3 cells. Pretreatment of either normal or tumor PC-3 cells by resveratrol increases somewhat the uptake of [^{3}]H-resveratrol.

Task 2. To identify and characterize target proteins of resveratrol in prostate cancer cells.

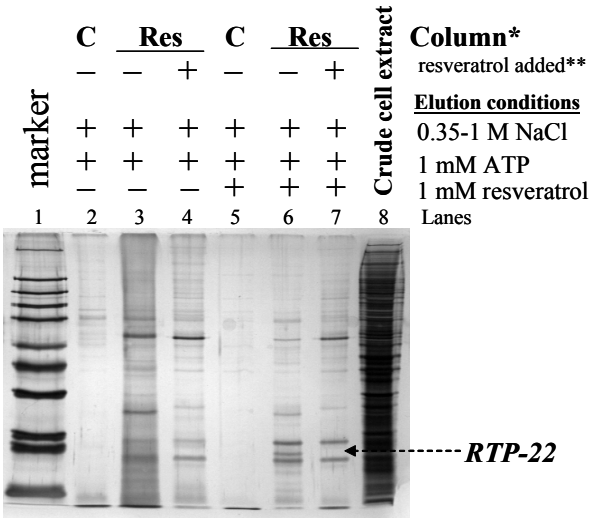
Task 2a. To develop resveratrol-based affinity columns.

Task 2b. To analyze proteins capable of specific interaction with resveratrol.

Response to technical issues on p2 the first and second concerns from reviewer.
Reviewer's comment: "Heedless of these results, the PI uses even more resveratrol (25 μM) for further experiments."
"Second, the PI does not explain his rationale for using resveratrol-treated cells for "LSP" (apparently, another name for affinity chromatography). Nevertheless, he does state, "It is evident that treatment with 25 μM resveratrol resulted in increased expression of several proteins…."
Response: Although one could limit studies aiming to demonstrate resveratrol binding and target proteins RTPs to only untreated CaP cell extracts, we considered that it was prudent to extend and include studies to resveratrol-treated CaP cells as well, since numerous studies (8-14), including those illustrated in an earlier section of this report, have repeatedly shown that resveratrol dose-dependently elicits multiple gene and protein responses. These observations lend credence to the possibility that this polyphenol affects cellular proteins RTPs in untreated and resveratrol-treated cells in ways that might be manifested as significant changes in target protein profiles. To test this possibility, we included cells treated with 25 μM resveratrol, which we consider a reasonably good representation of doses used in most *in vitro* published studies (dose range: 10-100 μM resveratrol).
Response to technical issues on p2 the second concerns from reviewer.

"However, the experiments from which he draws this conclusion are missing a number of controls, the most important one being an unmodified column (no resveratrol) to control for nonspecific binding of proteins. Other controls that should have been included are input or flow-through controls."

Response: The resveratrol affinity column was prepared according to the following procedure: One gram of epoxy-activated agarose was suspended in ice-cold water for 5 minute and washed extensively to remove preservatives. Resveratrol (23 mg) dissolved in 2.5 ml of 0.1 M NaOH was added to 1 ml of resuspended epoxy-activated agarose, followed by an overnight incubation at room temperature to allow chemical coupling of resveratrol to the resin. To stop the reaction, 6 ml of 1 M sodium acetate buffer (pH 5.0) containing 1 mM dithiothreitol (DTT) was added to the mixture to neutralize unreacted epoxy groups and prevent further oxidation of resveratrol. Immobilized resveratrol resin was washed successively with 0.1 M sodium acetate, pH 5.0, containing 1 mM DTT and 70%, 30%, 10% and 0% ethanol, respectively. Controls consisted of mock-treated beads (identical procedure except that no resveratrol was added) or beads immobilized with tyrosine as the ligand. Notably, the above-described method included controls mentioned by the reviewer (see new Fig 1 of this report, for results of fractionation of PC-3 cell extract on resveratrol affinity column).



*C: control resin; Res: resveratrol affinity resin
**: 1 mM resveratrol added during binding to the column

New Fig 1. Isolation and identification of resveratrol target proteins (RTPs). (A) Demonstration of binding of specific proteins from PC-3 cell extracts to resveratrol immobilized epoxy-activated agarose affinity columns. Cell lysates were fractionated on mock control column and resveratrol affinity column. Proteins were eluted from the column using sodium choloride (0.35M or 1M NaCl) followed by 1 mM ATP and 1 mM resveratrol. Elution profiles between control and resveratrol affinity column with or without elution by resveratrol and the identification of RTP-22. Note that RTP-22 was competed by adding resveratrol during binding to the column.

Response to technical issues on p3 the second concerns from reviewer.

"In addition, the PI does not provide any western data to support increased protein expression. If there is "increased expression of several proteins," it is curious that so many bind the resveratrol affinity column. Similar affinity chromatography with the related polyphenol Epigallocatechin gallate does not produce similar results."

Response: As mentioned earlier, increased and decreased expression of numerous proteins, in response to treatment by resveratrol, have been observed and reported by this laboratory and others as well (8-14). Examples include: upregulation of p53; down

regulation of PSA, both evident by data from western blot analysis. There is no a priori reason to assume that proteins whose expression is regulated by resveratrol would necessarily be ones corresponding to RTPs capable of binding to the affinity column we have developed, although such a possibility cannot be excluded. The reviewer is absolutely correct in pointing out that a significant number of proteins were found bound to the affinity column. It should be noted, however, that many of these proteins were displaced using increasing NaCl concentrations, still others were eluted with ATP, and only a few distinct ones were eluted with resveratrol. We focused on proteins displaced from the affinity column only with the use of high concentration of resveratrol (1 mM).

Response to technical issues on p3 the second concern from reviewer.
"Similar affinity chromatography with the related polyphenol Epigallocatechin gallate does not produce similar results."
Answer: We did not make attempts to generate affinity columns tagged with other polyphenols, e.g., epigallocatechin gallate (EGCG). Conceivably such columns, if generated, would be expected to bind proteins with specificity for EGCG and therefore may have little to no overlap with ones that bound to resveratrol affinity column we have developed.

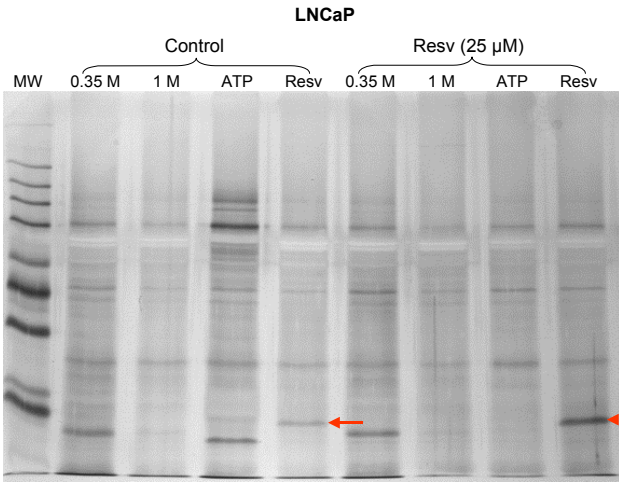
Response to technical issues on p3 the third concern from reviewer.
"Third, the PI claims that the estrogen and aryl hydrocarbon receptors elute under less stringent conditions than NQO2 but also claims they elute at 1 M NaCl, which is typically high stringency. Since he did not report using higher stringency, it is difficult to interpret these results."
Response: The reviewer is right, typically 1M NaCl is a more stringent condition for displacing proteins from affinity columns. As alluded to earlier, our goal was to isolate protein(s) having the highest and selective affinity for resveratrol (low Kd). To this end, we developed the sequential elution protocol by first using increase concentration of NaCl from 0.35M up to 1M to displace proteins with lower affinity for resveratol. This elution step was followed by a more stringent condition of elution with ATP. The final elution step used high concentration of resveratrol (1 mM) and was designed to select for proteins with significantly higher affinity for resveratrol. In this purification scheme, proteins eluted with 1M NaCl were regarded as being less tightly binding, compared to proteins that eluted with 1 mM resveratrol. Using this scheme, we have observed that estrogen and aryl hydrocarbon receptors were both bound to the resveratrol affinity column, and were quantitatively displaced with 1 M NaCl (data not shown) which was a considerably less stringent condition than that used for dihydronicotinamide riboside:quinone reductase (NQO2). Notably, NQO2 was eluted from the resveratrol affinity column only when 1 mM resveratrol was included in the eluting buffer.

Response to technical issues on p3 the last concern from reviewer.
"Last, the majority of the resveratrol would appear to be in the membrane/organelle cellular fraction. Membrane-bound proteins are notoriously difficult to isolate by affinity chromatography and important components may have been missed. The PI does not address this issue."

Response: As illustrated by results in Tables 1-3, resveratrol can be taken into normal and prostate tumor cells, albeit with a definite time lag and rather inefficiently. To better define the cellular fate of [^{3}]H-resveratrol taken in, we proceeded to fractionate cell extracts into various compartments, reasoning that this approach might provide us with the appropriate "window" to track the fate of [^{3}]H-resveratrol and, by extrapolation, identity of proteins it interacts with. As the reviewer noted, the majority of the [^{3}]H-resveratrol can be traced to the membrane/organelle fraction; membrane-bound proteins are notoriously difficult to isolate since they typically represent a very small portion of total cell extracts. In recognition of this technical challenge, we decided to first concentrate our efforts in identifying cytosolic RTPs as they are likely to represent the majority of proteins in whole cells. The knowledge and experience we have gained in fractionating cytosolic extracts hopefully will enhance our chance in tracking and purifying RTPs in the membrane/organelle or nuclear fractions in the future.

Task 2c. To purify resveratrol-binding proteins from LNCaP and PC-3 cells.

LNCaP cell extracts were similarly fractionated on resveratrol affinity column to identify and purify resveratrol targeting proteins RTPs (see new Fig 2 in this report). In addition, we also investigated the effects of resveratrol on RTP profiles displayed on resveratrol affinity columns. In these studies, LNCaP cell were first treated for 2 days with 25 μM resveratrol. Lysates were prepared from control and treated LNCaP cells, and separately applied and fractionated on resveratrol affinity column. A representative RTP profile from cytoplasmic extracts of control and resveratrol treated LNCaP is depicted in new Figure 2. Significant differences in silver stained patterns were observed in 1M ATP and resveratrol eluted fractions. Identity of some of these proteins remains to be determined.



New Fig 2. Identification of resveratrol interacting proteins (RTPs) in control and resveratrol treated LNCaP cells. Demonstration of binding of specific proteins from LNCaP cell extracts to resveratrol tag affinity resins. Cell lysates fractionated according to the scheme in Fig. 1 and eluted with 0.35 M, 1 M NaCl, ATP and 1 mM resveratrol revealed the presence of proteins bound to resveratrol affinity column. Elution profiles between control and 25 μM resveratrol treated LNCaP cell extracts are shown and the possible RTP is indicated by arrow.

Task 2d. To identify resveratrol-binding proteins using mass spectrometry.

Currently, dihydronicotinamide riboside quinone reductase 2 (NQO2) is the first RTP identified using mass spectrometry. We are in the process of possibly identifying another RTP.

KEY RESEARCH FINDINGS

- [^{3}H]resveratrol can be taken into normal and prostate tumor cells, albeit with a definite time lag and rather inefficiently.

- Uptake of [^{3}H]resveratrol is more robust in hormone-refractory human prostate PC-3 cells, compared to androgen-dependent LNCaP cells.

- Pretreatment of PC-3 cells for 2 days with 2 μM resveratrol results in a 2.7-fold increase in uptake of [^{3}H]resveratrol, compared to untreated PC-3 cells.

- Using fractionation procedures that separate cell extracts into cytosol, organelle, nucleus and cytoskeleton, we observed that the majority of radioactive resveratrol in both untreated and 2 day resveratrol-treated cells, can be traced to the organelle fraction.

- By chemically coupling resveratrol to epoxy-activated agarose to form a resveratrol affinity column, we have been able to isolate and identify dihydronicotinamide riboside quinone reductase 2 as novel resveratrol targeting protein in prostate cancer cells. Resveratrol affinity column chromatography also may lead to the isolation of additional resveratrol targeting proteins the identify of which remains to be fully elucidated. Salient features of this innovative and facile development, which we have named *ligand (resveratrol)-capture proteomics,* include significant attributes listed below.
    - Enable detection and analysis of low abundance proteins that often are challenging to visualize and characterize by conventional biochemical methods.
    - Generate RTP profiles characteristic of androgen-dependent LNCaP and androgen-independent PC-3 cells

REPORTABLE OUTCOMES

A manuscript summarizing the findings in this report is in preparation. In addition, we are also considering to submit an IDEA grant to the U.S. Army Medical Research and Material Command that builds on the observations reported herein.

Summary

We have made significant progress towards defining the identity and nature of cellular proteins, denoted RTPs, that specifically interact with resveratrol using normal and cancerous prostate cells. The identity of a specific RTP has been elucidated as quinone reductase 2. A second RTP is in the process of being purified and characterized.

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